Inhibiting Glycogen Synthase Kinase-3 Decreases 12-O-Tetradecanoylphorbol-13-Acetate-Induced Interferon-γ-Mediated Skin Inflammation

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ABSTRACT

Glycogen synthase kinase-3 (GSK-3) facilitates interferon (IFN)-γ signaling. Because IFN-γ is involved in inflammatory skin diseases, such as psoriasis, the aim of this study was to investigate the pathogenic role of GSK-3 in 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced IFN-γ-mediated skin inflammation. TPA (3 μg per ear) induced acute skin inflammation in the ears of C57BL/6 mice, including edema, infiltration of granulocytes but not T cells, and IFN-γ receptor 1-mediated deregulation of intercellular adhesion molecule 1 (CD54). TPA/IFN-γ induced GSK-3 activation, which in turn activated signal transducer and activator of transcription 1. Inhibiting GSK-3 pharmacologically, by administering 6-bromoindirubin-3’-oxime (1.5 μg per ear), and genetically, with lentiviral-based short-hairpin RNA, reduced TPA-induced acute skin inflammation but not T-cell infiltration. It is noteworthy that inhibiting GSK-3 decreased TPA-induced IFN-γ production and the nuclear translocation of T-box transcription factor Tbx21, a transcription factor of IFN-γ, in CD3-positive T cells. In chronic TPA-induced skin inflammation, inhibiting GSK-3 attenuated epidermis hyperproliferation and dermis angiogenesis. These results demonstrate the dual role of GSK-3 in TPA-induced skin inflammation that is not only to facilitate IFN-γ signaling but also to regulate IFN-γ production. Inhibiting GSK-3 may be a potential treatment strategy for preventing such effects.

Introduction

Skin provides the largest immunity barrier because disordered immune responses result in a large variety of inflammatory skin diseases, including psoriasis (Kupper and Fuhlbrigge, 2004). The causes of psoriasis can be genetic, infectious, or environmental stimuli (Nestle et al., 2009; Cojocaru et al., 2010). Cytokines released from Th1 and Th17 cells are believed to facilitate immunopathogenesis in immune-mediated psoriasis by inducing keratinocytes to generate inflammatory antimicrobial peptides, cytokines, and chemokines (Nestle et al., 2009; Zaba et al., 2009). Other investigations of psoriasis have focused primarily on the pathogenic role of Th17 cells (Krueger et al., 2007; Zheng et al., 2007; Steinman, 2010). However, the levels of interferon (IFN)-γ in serum are positively correlated with disease severity in psoriasis (Szegedi et al., 2003; Abdallah et al., 2009; Takahashi et al., 2010). It is noteworthy that IFN-γ-regulated gene expression is pivotal in psoriasis, and IFN-γ receptors (IFNGRs) 1 and 2 are overexpressed in psoriatic peripheral blood mononuclear cells; in addition, the activation of signal transducer and activator of transcription (STAT) 1 has been identified in psoriatic skin lesions (Yao et al., 2008). These results suggest an important role of IFN-γ in psoriasis.

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Abbreviations: IOD, integrated optical density; IL, interleukin.

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tant role for IFN-γ, a Th1 cytokine, in the progression of skin inflammation.

IFN-γ belongs to a family of type II IFN cytokines that are released from Th1, natural killer, and natural killer T cells, and it promotes antimicrobial responses, antigen processing, inflammation, growth suppression, cell death, tumor immunity, and autoimmunity (Platanias, 2005; Schoenborn and Wilson, 2007; Saha et al., 2010). After binding to IFNGR, IFN-γ activates Janus kinase (Jak) 1 and Jak2 sequentially. Jak1 then induces STAT1 binding to IFNγR1 followed by Jak2-mediated STAT1 phosphorylation at Tyr701 (Bach et al., 1997; Ramana et al., 2002). The activated STAT1 translocates to the nucleus and is necessary for the transactivation of proinflammatory molecules, including tumor necrosis factor-α, IFN-γ-induced protein 10, monocyte chemotactic protein-1, and regulated upon activation, normal T-cell expressed, and intercellular adhesion molecule 1 (CD54), but not the anti-inflammatory cytokine interleukin-10 (Donnelly et al., 1995; Schroder et al., 2004; Hu et al., 2006). IFN-γ also triggers inducible nitric-oxide synthase expression to facilitate generation of nitric oxide (Lorsbach et al., 1993; Gao et al., 2002). In addition to STAT1, IFN regulatory factor 1 (IRF-1) and nuclear factor-κ-light-chain enhancer of activated B cells are activated and are involved in typical IFN-γ signaling and inflammation (Ramana et al., 2002; Schroder et al., 2004; Kai et al., 2010). Furthermore, the pathogenic role of IFN-γ signaling has been demonstrated in murine psoriasis-like skin inflammation (Arakura et al., 2007; Sarra et al., 2011).

Glycogen synthase kinase-3 (GSK-3), a serine/threonine kinase, positively regulates multiple inflammatory diseases, including sepsis, arthritis, colitis, and multiple sclerosis, by influencing several critical transcription factors, such as nuclear factor-κB, nuclear factor of activated T cells, and STATs (Beurel et al., 2010; Kai et al., 2010; Wang et al., 2011). Other studies suggest that GSK-3 facilitates IFN-γ-mediated inflammation by mediating STAT1 activation (Tsai et al., 2009). Furthermore, GSK-3 facilitates the Con A-induced IFN-γ-mediated immune hepatic injury by promoting STAT1 activation and the nuclear translocation of T-box transcription factor Tbx21 (T-bet) (Tsai et al., 2011). We therefore hypothesized that GSK-3 may facilitate IFN-γ-mediated skin inflammation. The model of inflammation used in this study was cutaneous inflammation induced by using 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment on rodent ears. TPA-induced acute skin inflammation causes ear edema and inflammatory infiltration. Long-term treatment with TPA induces skin epidermal hyperplasia, dermal immune cell infiltration, and angiogenesis (De Vry et al., 2005; Park et al., 2011). In this study, we investigated the role of GSK-3 in TPA-induced skin inflammation in vivo and the role of IFN-γ signaling in epidermal keratinocytes in vivo and in vitro.

Materials and Methods

Drugs and Reagents. TPA and GSK-3 inhibitor 6-bromoindirubin-3′-oxime (BIO) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human IFN-γ was obtained from PeproTech (Rocky Hill, NJ). 4′,6-Diamidino-2-phenylindole (DAPI) and mouse anti-β-actin monoclonal antibody were obtained from Sigma-Aldrich. Alexa Fluor 488-labeled anti-mouse CD45, CD3ε, Gr-1, CD84, and CD31 and anti-mouse T-bet were obtained from BioLegend (San Diego, CA). Alexa Fluor 488- and 594-labeled and horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit, goat anti-rat, and donkey anti-goat IgG were from Invitrogen (Carlsbad, CA). The antibody for mouse STAT1, phospho-glycogen synthase (pGS; Ser641), and GSK-3β were obtained from Cell Signaling Technology (Danvers, MA). Anti-mouse phospho-STAT1 (Tyr701), phospho-GSK-3α/β (Tyr279/Tyr216), and Ki-67 antibodies were obtained from Abcam Inc. (Cambridge, MA). Anti-GSK-3α/β was obtained from Santa Cruz Bio-technology, Inc. (Santa Cruz, CA). Rat monoclonal antibody targeted against mouse IFN-γ was obtained from Bender MedSystems (San Bruno, CA). Anti-mouse Gr-1-neutralizing antibody (clone RB6-8C5) was obtained from eBiScience (San Diego, CA).

Animal Treatment. The 8- to 12-week-old progeny of wild-type C57BL/6 mice and IFNγR1-deficient [Ifngr1(−/−)] mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). They were fed standard laboratory chow and water ad libitum in the Laboratory Animal Center of National Cheng Kung University. The animals were raised and cared for according to the guidelines set by the National Science Council of Taiwan. The experimental protocols adhered to the rules of the Animal Protection Act of Taiwan and were approved by the Laboratory Animal Care and Use Committee of National Cheng Kung University. As shown previously, to establish the TPA-induced acute skin inflammatory model, 30 μl of phosphate-buffered saline (PBS; pH 7.4), dimethyl sulfoxide (DMSO) diluted in PBS as a solvent control, and 50 μg · ml⁻¹ TPA (Sigma-Aldrich) dissolved in solvent control (3 μg per ear) with or without the optimal dosage of 25 μg · ml⁻¹ of the GSK-3 inhibitor BIO (Sigma-Aldrich) (Tsai et al., 2011) dissolved in solvent control (1.5 μg per ear) were dropped into 1-cm² pieces of 100% spunlace rayon that was 0.5 mm thick, and two pieces of rayon were then placed against the inner and outer surface of each ear for 1 h as one treatment. This chronic skin-inflammation model is modified from that developed by De Vry et al. (2005) (Park et al., 2011). For chronic TPA-induced cutaneous inflammation, we spaced the treatment over 3 days and repeated the treatment six times over 15 days. On day 16, the mice were administered a lethal overdose of intraperitoneal pentobarbital (200 mg · kg⁻¹), and their ear tissue was harvested at the indicated times postinjection.

Cell Culture and Cytotoxicity Assay. Human keratinocyte HaCaT cells were provided by Chia-Yu Chi, Division of Clinical Research, National Health Research Institutes, Taiwan. The cells were routinely grown on plastic in RPMI 1640 (Invitrogen) containing 10% heat-inactivated fetal bovine serum (Invitrogen), 50 U penicillin, and 50 μg · ml⁻¹ streptomycin and maintained in a humidified atmosphere containing 5% CO₂.

Immunohistochemistry and Immunostaining. All tissue sections were deparaffinized, rehydrated, incubated with 3% H₂O₂ in methanol for 15 min, and subjected to heat-induced antigen retrieval by autoclaving them for 5 min in 10 mM citric acid buffer, pH 6.0. After two washes in PBS, the tissue sections and cells were mixed with primary antibodies in antibody diluents (Dako North America, Inc., Carpinteria, CA) and incubated at 4°C overnight. The next day, the samples were washed with PBS and then incubated with or without horseradish peroxidase- and Alexa Fluor 488- or 594-labeled secondary antibodies at room temperature for 1 h. For immunohistochemistry, sections were washed with PBS, developed using AEC substrate (Dako North America, Inc.), counterstained with hematoxylin (Sigma-Aldrich), and visualized with an inverted microscope (IX71; Olympus, Tokyo, Japan). For immunostaining, sections and cells were washed with PBS and visualized with a DP70 camera and a fluorescent right BX51 microscope (Olympus) or a linear sequential C1Si laser scanning head and an Eclipse TE2000-E inverted microscope (Nikon, Tokyo, Japan). DAPI (1:200; Sigma-Aldrich) was added for nuclear counterstaining and applied at room temperature for 10 min. The confocal images were captured in a single x-y scan (100× enlarged) or in three dimensions (150× enlarged; 0.5 μm per z step).
GSK-3 Facilitates Skin Inflammation

IFNGR1 Is Required for TPA-Induced Acute Skin Inflammation. IFN-γ is associated with immunopathogenesis in psoriasis (Nestle et al., 2009; Takahashi et al., 2010). Using TPA (3 μg per ear)-induced acute skin inflammation in C57BL/6 mice, as shown previously (Park et al., 2011), we found that TPA induced STAT1 activation, which was detected by using STAT1 phosphorylation at Tyr701 and protein expression in the epidermis and dermis of wild-type but not Ifngr1−/− mice (n = 3 per group; Fig. 1A). H&E staining (Fig. 1B) showed that ear thickness was significantly (215.9 ± 48.8, DMSO only versus 503.2 ± 142.5, TPA; p < 0.001) increased in TPA-treated wild-type mice but was significantly (503.2 ± 142.5, wild type versus 382.5 ± 59.5, Ifngr1−/−; p < 0.05) attenuated in Ifngr1−/− mice compared with the DMSO-treated group (Fig. 1C). Furthermore, IFNGR1 deficiency significantly attenuated the TPA-induced infiltration of leukocytes (367.1 ± 204.8, wild type, versus 165.1 ± 28.8, Ifngr1−/−; p < 0.05; Fig. 1D) and granulocytes (238.2 ± 156.5, wild type versus 76.3 ± 49.8, Ifngr1−/−; p < 0.01; Fig. 1F) and the expression of adhesion molecule CD54 (206.9 ± 78.6, wild type versus 125.1 ± 72.9, Ifngr1−/−; p < 0.05; Fig. 1G) but not the infiltration of CD3+ T cells (Fig. 1E). These results indicate that IFN-γ signaling is essential for TPA-induced acute skin inflammation including edema, granulocyte infiltration, and CD54 expression but not T cell infiltration.

TPA Activates GSK-3 and Induces GSK-3-Regulated IFN-γ Signaling. We demonstrated previously that GSK-3 facilitates IFN-γ/STAT1 signaling (Tsai et al., 2009). We therefore hypothesized that GSK-3 facilitates TPA-induced acute skin inflammation. AEC-based immunohistochemistry (Fig. 2A) showed that TPA-induced GSK-3 activation (1.0 ± 0.4, DMSO only versus 3.1 ± 1.9, TPA; p < 0.05; Fig. 2B), using a semidry electroblotting system. After blocking with 5% skim milk in PBS, membranes were incubated at 12,000 rpm at 4°C for 20 min. Lysates were boiled in sample buffer for 5 min. Proteins were then subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA) with a 1/1000 dilution of primary antibodies at 4°C overnight. Membranes were then washed using 0.05% PBS-Tween 20 and incubated with a 1/5000 dilution of horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. After washing, membranes were soaked in an ECL solution (PerkinElmer Life and Analytical Sciences, Boston, MA) for 1 min and then exposed to film (BioMax; Eastman Kodak, Rochester, NY). Luciferase Reporter Assay. For the luciferase reporter assay, the cells were transiently cotransfected by using a GeneJammer reagent (Agilent Technologies, Santa Clara, CA) with an IRF1 promoter-driven luciferase reporter (0.2 μg) and 0.01 μg of Renilla luciferase-expressing plasmid (pRL-TK; Promega). Twenty-four hours after the transfection, the cells were treated with IFN-γ for 3 h, lysed, and then harvested for luciferase and Renilla measurement by using a luciferase assay kit (Dual-Glo; Promega). For each lysate, the firefly luciferase activity was normalized to the Renilla luciferase activity to assess transfection efficiency. Granulocyte Depletion. For the depletion of granulocytes, each mouse received intraperitoneal injections of 50 μg of monoclonal rat-anti-mouse Gr-1 antibody (clone RB6-8C5; eBioscience) in 100 μl of PBS (Jablonska et al., 2010).

Statistical Analysis. Values are expressed as the mean ± S.D. The groups were compared by using Student’s two-tailed unpaired t tests or a one-way analysis of variance in Prism version 5 (GraphPad Software Inc., San Diego, CA). A value of p < 0.05 was considered to be statistically significant.
which was detected as GSK-3β phosphorylation at Tyr216, an active residue of GSK-3β, and glycogen synthase phosphorylation at Ser641, a substrate of GSK-3, as shown previously (Tsai et al., 2011), in the epidermis (1.4 ± 0.5, DMSO only versus 6.5 ± 3.1, TPA; p < 0.05; Fig. 2C) and dermis (0.9 ± 0.4, DMSO only versus 4.2 ± 2.8, TPA; p < 0.05; Fig. 2D). To verify the role of activated GSK-3, GSK-3β was inhibited by using the inhibitor BIO, which binds within the ATP-binding pocket of GSK-3. The results showed that treatment with BIO (1.5 μg per ear, the maximum dosage selected from three dilutions; data not shown), reduced TPA-induced STAT1 activation and expression in the epidermis but not the dermal infiltrate of wild-type mice (Fig. 2E). We next investigated the role of GSK-3 in epidermal keratinocytes in response to IFN-γ. The expression of GSK-3α/β was knocked down in HaCaT human keratinocytes by using a lentiviral-based shRNA approach (Fig. 2F). A luciferase reporter assay for IRF-1 promoter transactivation showed that the knockdown of GSK-3α/β, consistent with BIO (2.5 μM) treatment, significantly (11.6 ± 5.2, DMSO only versus 5.2 ± 2.8, BIO, 5.5 ± 1.8, shGSK-3-α, and 4.2 ± 1.3, shGSK-3β; p < 0.05) inhibited IFN-γ (10 ng·ml⁻¹) signaling (Fig. 2G). These results demonstrate that TPA induces GSK-3 activation in the epidermis and leads to GSK-3 regulation of IFN-γ signaling.

**GSK-3 Facilitates TPA-Induced Acute Skin Inflammatory Responses.** BIO was used to investigate whether inhibiting GSK-3 reduces TPA-induced acute skin inflammatory responses. It is noteworthy that inhibiting GSK-3 attenuated TPA-induced ear swelling (Fig. 3A and Supplemental Fig. 1A). Furthermore, ears treated with BIO displayed an attenuation of the TPA-induced increased ear thickness (501.0 ± 138.9, TPA only versus 299.0 ± 41.0, TPA + BIO; p < 0.05; Fig. 3B), the infiltration of leukocytes (511.7 ± 203.1, TPA only versus 125.5 ± 84.7, TPA + BIO; p < 0.01; Fig. 3C) and granulocytes (238.2 ± 156.5, TPA only versus 102.5 ± 77.1, TPA + BIO; p < 0.01; Fig. 3E), and the expression of CD54 (240.1 ± 94.1, TPA only versus 140.6 ± 93.5, TPA + BIO; p < 0.01; Fig. 3F) but not CD3+ T cell infiltration (Fig. 3D). Thus, BIO induces the infiltration of granulocytes. The depletion of granulocytes, using an anti-Gr-1 antibody, suggests that granulocytes may be pathogenic in TPA-induced acute ear edema (Supplemental Fig. 1, B and C). These results demonstrate that GSK-3 activation is pathogenic for TPA-induced acute skin inflammation, including edema, granulocyte infiltration, and CD54 expression but not T cell infiltration.

**Epidermal GSK-3 Facilitates TPA-Induced Edema and Granulocyte Infiltration.** To further clarify the role of GSK-3 in epidermal keratinocytes in vivo, we created a local knockdown of GSK-3β by using lentiviral-based shRNA interference in the ears of C57BL/6 mice. Our GSK-3 immunohistochemical results (Fig. 4A) show that GSK-3 knockdown reduces GSK-3β expression in the epidermis markedly (4.9 ± 1.5, shLuc versus 2.6 ± 0.9, shGSK-3β; p < 0.01; Fig. 4B). Analysis of histological morphology (Fig. 4C) revealed that the epidermal knockdown of GSK-3β significantly attenuated both TPA-induced ear edema (515.3 ± 73.6, shLuc versus 416.6 ± 39.9, shGSK-3β; p < 0.05; Fig. 4D) and granulocyte infiltration (272.6 ± 88.6, shLuc versus 169.3 ±
GSK-3 Facilitates Skin Inflammation

Fig. 2. TPA induces GSK-3 activation followed by GSK-3-regulation of IFN-γ signaling. A, AEC-based immunohistochemistry of phospho-GSK-3β Tyr216 (pGSK-3) and phospho-GSK Ser421 (pGSK) of the ears of wild-type C57BL/6 mice (n = 5 per group) individually soaked for 1 h with PBS, DMSO (solvent control), or TPA (3 μg per ear) for 8 h. Representative images from repeated experiments are shown. B to D, the expression of total GSK-3β (B), epidermal GSK-3β (C), and dermal GSK-3β (D) was measured from captured fields. Data are shown as the mean ± S.D. of three individual experiments compared with the normalized untreated group (PBS only). *, p < 0.05 compared with the DMSO-treated group. E, AEC-based immunohistochemistry of phospho-STAT1 Tyr701 (pSTAT1) and STAT1 of the ears of wild-type C57BL/6 mice (n = 5 per group) individually soaked with PBS, DMSO (solvent control), or TPA (3 μg per ear) with or without GSK-3 inhibitor BIO (1.5 μg per ear) for 8 h. A representative data set obtained from repeated experiments is shown. F, GSK-3α/β was silenced in HaCaT human keratinocytes by using lentiviral-based short hairpin RNA transfection (shGSK-3α/β clones 1 and 2). An shRNA targeting luciferase (shLuc) was used as a negative control. Western blotting was used to detect the expression of GSK-3α/β. β-Actin was used as an internal control. A representative data set obtained from repeated experiments is shown. G, the cells were then treated with IFN-γ (10 ng · ml⁻¹) or PBS. A luciferase reporter assay of promoter transactivation was used to detect the ratio of IRF-1 regulation to Renilla in cells treated with IFN-γ. p < 0.05 compared with PBS. #: p < 0.05 compared with IFN-γ. Cells treated with BIO (2.5 μM) were used as a positive control.

61.0, shGSK-3β; p < 0.01; Fig. 4E). These results imply that GSK-3 activation is key for TPA-induced edema and granulocyte infiltration.

GSK-3 Facilitates TPA-Induced IFN-γ Production in CD3-Positive T Cells by Regulating T-Bet Nuclear Translocation. The pathogenesis of immune-mediated psoriasis is thought to be T cell-mediated (Schön and Boehncke, 2005). Our findings show that GSK-3-regulated IFN-γ signaling is not involved in CD3-positive T cell infiltration. Immunohistochemistry for IFN-γ (Fig. 5A) showed that TPA markedly induced IFN-γ production at 4 h (13.3 ± 7.8, DMSO only versus 37.1 ± 10.1, TPA; p < 0.001; Fig. 5B) compared with the DMSO-treated group. However, treatment with BIO significantly (37.1 ± 10.1, TPA only versus 20.6 ± 7.2, TPA + BIO; p < 0.05) decreased TPA-induced dermal IFN-γ expression. GSK-3 is required for the nuclear translocation of T-bet (Hwang et al., 2005; Tsai et al., 2011). Immunostaining and confocal image analysis identified TPA-induced GSK-3 activation, which was determined by the phosphorylation of GSK-3 at Tyr216 in CD3-positive T cells (Fig. 5C). It is noteworthy that inhibiting GSK-3 decreased the TPA-induced T-bet nuclear translocation in CD3-positive T cells (Fig. 5D and Supplementary Fig. 2). These results demonstrate that GSK-3 activation in T cells facilitates IFN-γ production by facilitating T-bet nuclear translocation in TPA-induced acute skin inflammation.

GSK-3 Facilitates IFN-γ-Mediated Chronic TPA-Induced Psoriasis-Like Cutaneous Inflammation. To further clarify the role of GSK-3 in skin inflammation, we also assessed the protective effects of GSK-3 inhibition in a model of chronic TPA treatment (Fig. 6A). BIO treatment strikingly attenuated TPA-induced edema and epidermal swelling (Fig. 6B), ear thickness (539.1 ± 66.3, TPA only versus 327.7 ± 51.1, TPA + BIO; p < 0.01; Fig. 6C), and epidermal thickness (73.6 ± 15.1, TPA only versus 38.7 ± 4.6, TPA + BIO; p < 0.01; Fig. 6D). To investigate the role of GSK-3 in IFN-γ-regulated chronic TPA-induced cutaneous inflammation, immunostaining of Ki-67, a proliferation marker, and CD31, an endothelial cell marker, were used (Supplementary Fig. 3). The results showed that inhibiting GSK-3 significantly suppressed TPA-induced epidermal cell proliferation (206.8 ± 66.3, TPA only versus 90.7 ± 44.2, TPA + BIO; p < 0.05; Fig. 6E) and dermal angiogenesis (89.0 ± 28.1, TPA only versus 25.0 ± 12.5, TPA + BIO; p < 0.01; Fig. 6F). Immunostaining showed that chronic TPA treatment also induced GSK-3 activation (Fig. 6G). These results demonstrate that inhibiting GSK-3 attenuates chronic TPA-induced cutaneous...
inflammation including edema, epidermal proliferation, and angiogenesis.

Discussion

In this study, we used a murine model of TPA-induced skin inflammation to demonstrate that the activation of GSK-3 not only facilitates IFN-γ signaling in keratinocytes in vivo and in vitro but is also required for IFN-γ production in T cells by activating T-bet, which is an important transcription factor for IFN-γ. Furthermore, inhibiting GSK-3 provides partial cellular protection from TPA-induced acute edema, granulocyte infiltration, CD54 expression, and TPA-induced chronic epidermal proliferation and dermal angiogenesis. These findings are summarized briefly in

Fig. 3. Inhibiting GSK-3 decreases TPA-induced acute skin inflammation but not T cell infiltration. A, H&E staining was used to determine the ear thickness (shown in μm) in wild-type C57BL/6 mice (n = 3 per group) individually soaked with PBS, DMSO (solvent control), or TPA (3 μg per ear) with or without BIO (1.5 μg per ear) for the indicated time. B to F, at 8 h post-treatment, the thickness of ear sections (B) was determined from H&E-stained sections, and immunostaining for leukocytes using anti-CD45 (C), T cells using anti-CD3 (D), granulocytes using anti-Gr-1 (E), and CD54-expressing cells (F) was conducted. Data are shown as the mean ± S.D. of three individual experiments. **, p < 0.01; ###, p < 0.001 compared with the DMSO-treated group. #, p < 0.05; ##, p < 0.01 compared with the TPA-treated group. ns, not significant.

Fig. 4. Local knockdown of GSK-3 attenuates TPA-induced edema and granulocyte infiltration. Lentiviral-based shRNA (1.2 × 10^6 plaque-forming units of lentivirus per ear) was used to knock down epidermal GSK-3β in vivo in the ears of wild-type C57BL/6 mice, as described under Materials and Methods. A shRNA targeting luciferase (sh-Luc) was used as a negative control. After 24 h, mouse ears were soaked for 1 h with DMSO (solvent control) and TPA (3 μg per ear). A, AEC-based immunohistochemistry of GSK-3β was performed on the ear tissue of treated mice. B, the expression of GSK-3β was measured from captured fields. Data are shown as the mean ± S.D. of three individual experiments. ##, p < 0.01 compared with the shLuc group. C to E, ear sections were then examined for H&E staining (C), ear thickness (D), and immunostaining of granulocytes using anti-Gr-1 (E). Data are shown as the mean ± S.D. of three individual experiments. A representative data set obtained from repeated experiments is shown. ***, p < 0.001 compared with the DMSO-treated group. #, p < 0.05; ##, p < 0.01 compared with the shLuc group.
IFN-γ is an important proinflammatory cytokine for several types of psoriasis (Szegedi et al., 2003; Yao et al., 2008; Abdallah et al., 2009; Takahashi et al., 2010). To the best of our knowledge, this study provides the first evidence showing that IFNGR1 is required for TPA-induced skin inflammation. We speculate that these findings may explain the effects of aberrant IFN-γ production (Szegedi et al., 2003; Abdallah et al., 2009; Takahashi et al., 2010) and STAT1 activation in psoriatic lesions (Yao et al., 2008). It is noteworthy that inhibiting GSK-3 with BIO or lentiviral-based GSK-3β-targeted shRNA resulted in an inhibition of the TPA-induced skin inflammation. Mechanistically, GSK-3 not only facilitates IFN-γ signaling in keratinocytes but is also required for TPA-induced IFN-γ production independent of the interference with T cell infiltration. These findings are consistent with studies that suggest that GSK-3 facilitates IFN-γ-mediated inflammation by activating STAT1 (Tsai et al., 2009).

In addition, GSK-3 facilitates IFN-γ-mediated inflammatory activation by promoting STAT1 activation and the nuclear translocation of T-bet, which results in an increase in IFN-γ production. In addition to IFN-γ, the role of Th17 cell activation and differentiation in GSK-3 activation requires further investigation (Beurel et al., 2011). The role of Th17 responses in TPA-induced skin inflammation also requires further investigation. In addition to Th1-IFN-γ-mediated skin inflammation, we speculate that inhibiting GSK-3 may reduce Th17 responses.

Treatment with TPA induces GSK-3β activation in epidermal keratinocytes and infiltrated T cells. A limitation of this study is that it remains unclear whether TPA induces GSK-3 activation directly or indirectly through other mechanisms, such as damage-associated molecules. Previous studies suggest that the activation of GSK-3 by IFN-γ, IL-6, and granulocyte-macrophage colony-stimulating factor facilitates the activation of STAT1, STAT3, STAT5, and STAT6 (Tsai et al., 2011) and TPA-induced IFN-γ production independent of the interferon with T cell infiltration. These findings are consistent with studies that suggest that GSK-3 facilitates IFN-γ-mediated inflammation by activating STAT1 (Tsai et al., 2009).
It is therefore hypothesized that these cytokines determine TPA-mediated GSK-3 activation and could induce IFN-γ, IL-6, and granulocyte macrophage colony-stimulating factor expression (Oberg et al., 2001). Consistent with our previous studies (Lin et al., 2008; Tsai et al., 2009; Kai et al., 2010) and those of others (Beurel et al., 2010; Wang et al., 2011), treatment with IFN-γ causes GSK-3 activation followed by GSK-3-mediated STAT1 activation. We further verified that the molecular mechanism underlying IFN-γ-activated GSK-3 is the triggering of bioactive sphingolipid ceramide-mediated pathways (Tsai et al., 2009; Wang et al., 2011). We therefore hypothesize that the activation of GSK-3 by ceramide may be involved in TPA activation of T cells and IFN-γ-activated keratinocytes. The involvement of ceramide signaling in TPA-induced skin inflammation requires further investigation. In addition to GSK-3β, knockdown of GSK-3α, which is a functionally redundant GSK-3 under some conditions (Beurel et al., 2010; Wang et al., 2011), reduced IFN-γ signaling. Essentially, the pharmacological inhibition of GSK-3 by BIO did not exclude the involvement of GSK-3α, and it is possible that GSK-3α activation plays a role in TPA-induced skin inflammation.

Lithium, a nonselective inhibitor of GSK-3 that competes with magnesium ions, is commonly used for the treatment of bipolar disorders. Long-term oral lithium salts are associated with a variety of pharmacological side effects, including gastrointestinal pain, discomfort, diarrhea, tremor, polyuria, nocturnal urination, weight gain, edema, nephrogenic diabetes insipidus, hypothyroidism, hyperparathyroidism, hypercalcemia, and lithium-induced psoriasis (Grandjean and Aubry, 2009). However, the immunopathogenesis of lithium-induced psoriasis may be different from that of typical psoriasis (Knijff et al., 2005). A reduction in intracellular calcium release after lithium treatment is hypothesized as the possible mechanism underlying lithium-aggravated psoriasis (Fry and Baker, 2007). A recent study found that inhibiting GSK-3 provoked keratinocyte proliferation (Hampton et al., 2012); however, there are no reports showing that GSK-3 is inhibited in psoriatic lesions or inactive GSK-3 exacerbates psoriasis. Manipulating GSK-3 for psoriatic therapy requires further consideration, particularly with regard to the time period and dose of inhibitor treatment.
In conclusion, our study demonstrates that the inhibition of GSK-3 not only blocks IFN-γ production but also reduces IFN-γ-mediated STAT1 activation and inflammatory responses. Our results suggest that inhibiting GSK-3 by using pharmacological or genetic manipulations is a potential therapeutic strategy for IFN-γ-mediated skin inflammation not only via the inhibition of IFN-γ production but also the blockade of IFN-γ signaling, granulocyte infiltration, CD54 expression, edema, epidermal hyperplasia, and angiogenesis. The findings of this study may provide insight for future preclinical or clinical studies that use more comparable models of IFN-γ and GSK-3-mediated human skin diseases.

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Authorship Contributions


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GSK-3 Facilitates Skin Inflammation 133